Mass Spectrometry Based Proteomics

Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

Mod 01 Lecture Number 03

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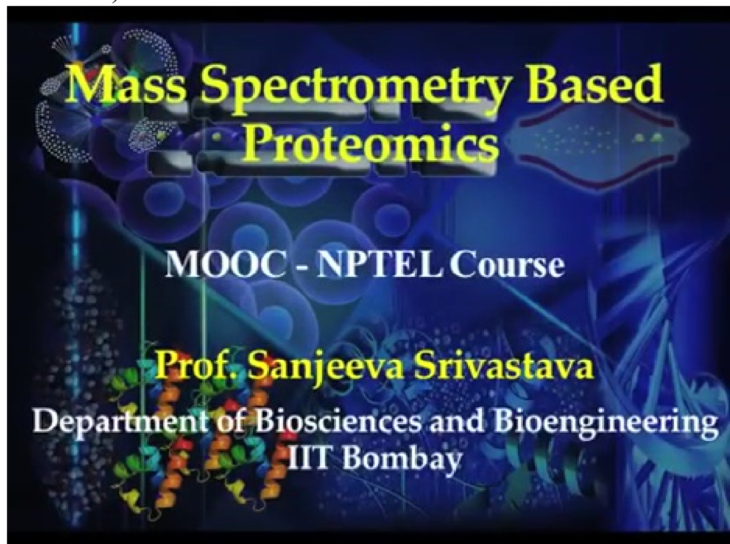

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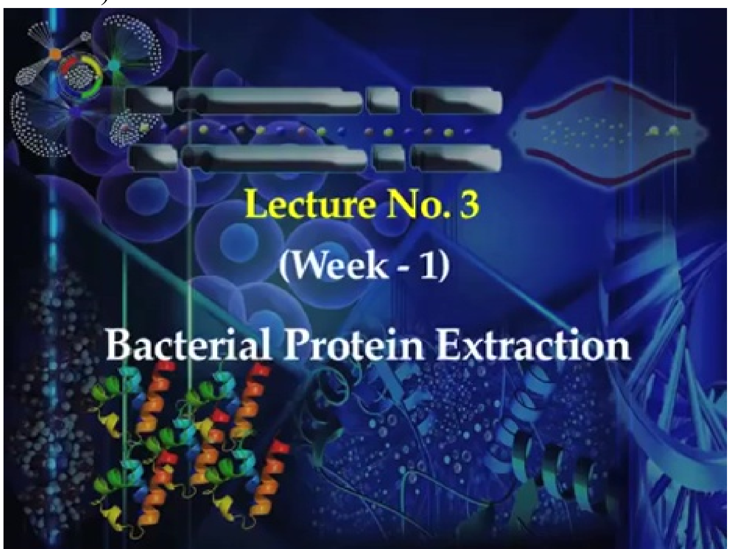

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Topics to be Discussed Today

Cell Lysis

TRIzol protein extraction method

TRIzol Method: Animation

TRIzol Method: Lab Demonstration

Protein Quantification

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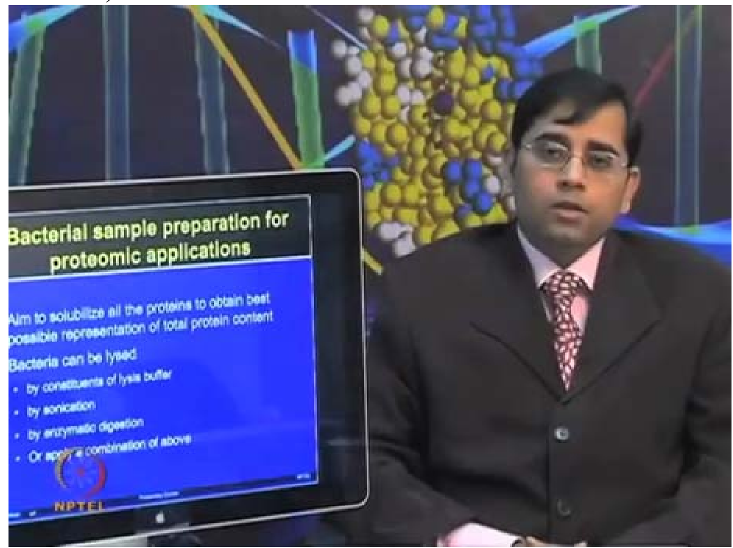

Let us talk about sample preparation for bacterial proteomics application. Similar to other samples, aim is to solubilize all the proteins because if you are aiming for a proteome level analysis, you would like to obtain all the proteins so that you can represent all the possible proteins on gel map or by using a LC MS approach.

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Now bacterial lysis is often very challenging for different type of bacteria but people have tried different type of lysis methods such as constituents of lysis buffer can be used for lysis.

Sonification is very efficient. People have also used enzymatic digestion methods as well as sometimes, a combination of these methods have been applied for bacterial lysis. We talked about different types of lysis methods.

We talked about how to prevent the proteolysis during the lysis, different types of protein prefactionation methods. Then we talked about protein precipitation, protein solubilization and how to remove various types of interfering components.

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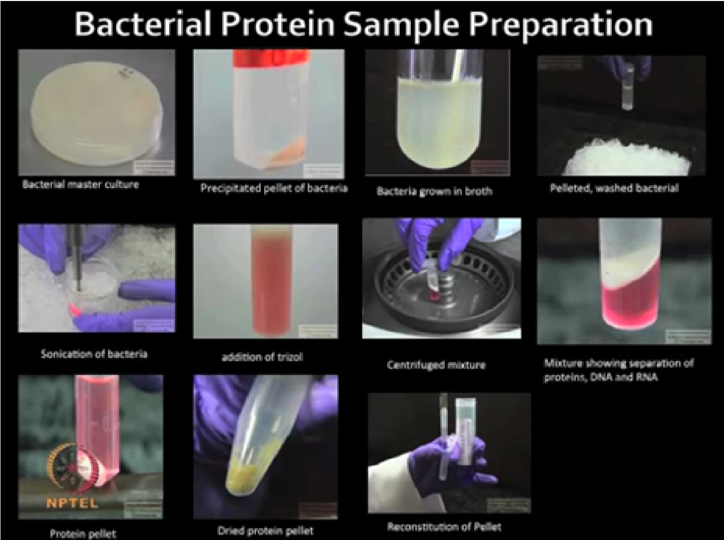

On the slide, various steps which are involved in bacterial protein sample preparation. So now let's elaborate on this and continue. Different types of sample preparation strategies which people apply for bacterial protein extraction; these are all reported methods available in the literature.

I have just compiled it for your brief overview. For detailed each of the protocol, you can refer to the detailed publications

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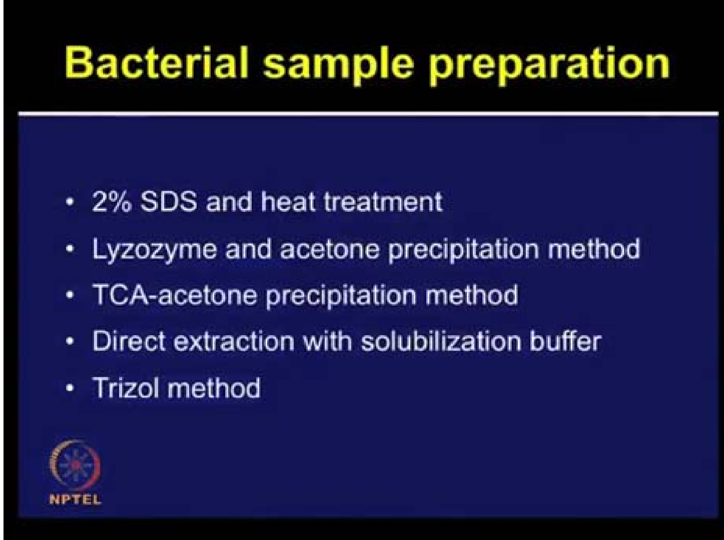

So 2% SDS and heat treatment has been applied for bacterial sample preparation. Similarly lysozyme and acetone precipitation method have been used. Trichloroacetic acid and acetone together have been effectively used for precipitation.

Direct protein extraction and solubilization in the solubilization buffer, this strategy has also been used and then a method, Trizol method has been used for bacterial sample preparation. I will elaborate on the Trizol method.

So, why we want to elaborate on Trizol method; because, this method provides you an opportunity for recovering DNA, RNA and proteins, all 3 major bio-molecules from the same sample. In the sequential extraction method one can obtain DNA, RNA and protein all by using this method.

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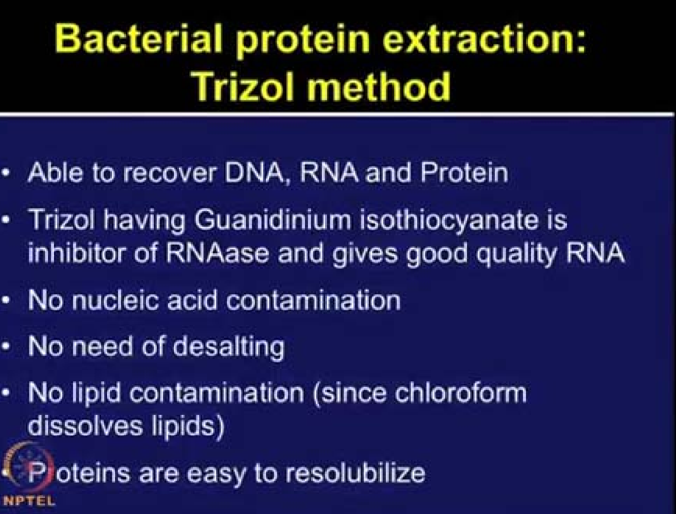

Trizol having guanidinium isothiocyanate is one of the inhibitors for RNase and it provides very good quality of RNA. This method also eliminates any possibility of nucleic acid contamination because you are already removing DNA and RNA.

Salts are also getting rid of using this method. There is no lipid contamination because chloroform is being used along with Trizol which dissolves the lipids. So, this method is able to get rid of various types of interfering components as well as different types of contaminants.

And finally, the proteins are easy to re-solubilize after extraction from the Trizol-based method. So this method is very useful for extracting bacterial and other proteome analysis.

Let me brief you about the procedure. So first, this is not the exact detail for the protocol. Just I am giving you few numbers for your reference. One can modify these depending on how much protein extract they want and based on their sample.

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Bacterial protein extraction: Trizol method

- Procedure:
- Add 1 ml trizol reagent to the bacterial suspension
- Add 200 ul chloroform to the mixture
- Vortex vigorously & incubate for 15 min at RT
- Centrifugation at 12000 g for 15 min

So you can start with 1 ml of Trizol reagent and add that in the bacterial suspension. Then add 200 micro liter of chloroform immediately to the same mixture. Vortex this mixture vigorously for 15 to 30 seconds and incubate it at the room temperature for 15 minutes. Centrifuge the sample at 12000 g for 15 minutes.

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Bacterial protein extraction (contd..)

- Carefully remove upper layer containing RNA using a micropipette
- To bottom layer, add 300 ul ethanol
- Centrifuge at 5000 g for 5 min to remove DNA
- Remove supernatant containing protein collect into a fresh tube
- In supernatant, add 4 volumes of chilled acetone and incubate for ~ 4 hrs at – 20^{0}C

NPTEL

After the centrifugation, remove the upper layer which contains RNA; and the bottom layer, you can add ethanol. Again you need to centrifuge at 5000 g for 5 minutes. This step will remove any DNA contamination.

Now in fact, this step can be used for keeping the material safe for further DNA extraction. Same applies to the previous step where you can collect the RNA which can be further used for RNA cleanup and preparation.

So, once you have removed the supernatant which contains the protein, then you can collect that in a fresh tube and in this supernatant you can add 4 volumes of chilled acetone. Incubate this mixture at -20 degrees for 4 to 6 hours.

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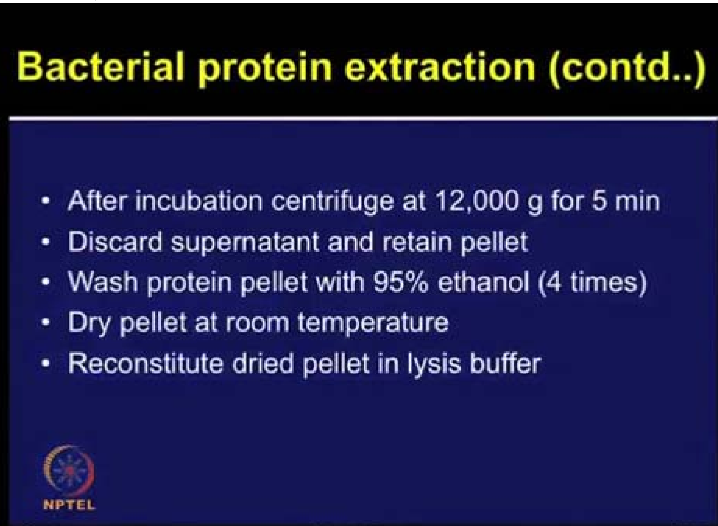

Once incubation is done, then start centrifugation step for 5 minutes. Discard the supernatant after centrifugation and retain the pellet. This pellet can be washed with 95% ethanol 3 to 4 times. Then dry this pellet at the room temperature and this pellet can be reconstituted in a buffer suitable for your analysis.

If you are going to perform two-dimensional electrophoresis you need to add the lysis buffer which contains urea, CHAPS, thiourea and different other components which we have talked previously.

Or if you want to analyze on SDS PAGE, you can add the Laemmli buffer. So depending upon your application, you can reconstitute the dried pellet in the right buffer composition

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. So let me show you the bacterial protein sample preparation in the interactive animations.

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For any bacterial proteome analysis, first you need to grow the bacterial cultures on algae media containing suitable antibiotic.

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Now proper 37 degrees temperature is provided for overnight or 6 to 8 hours of growth

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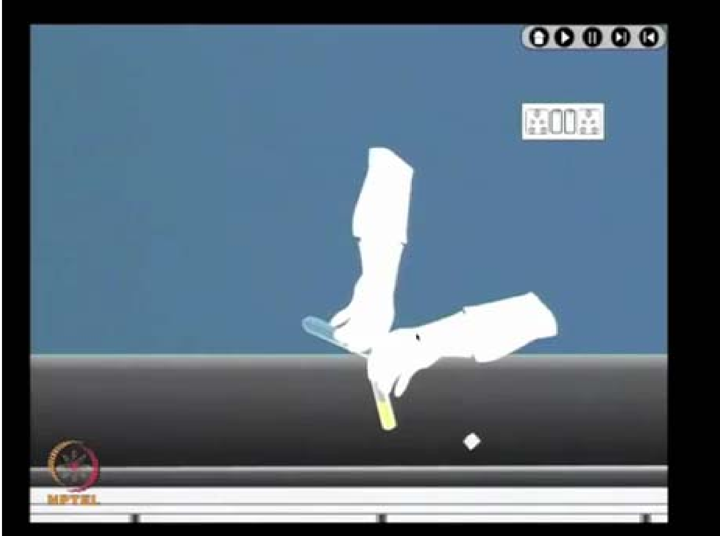

The overnight cultures are diluted with fresh LB…

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…and grown at 37 degrees for 6 to 8 hours.

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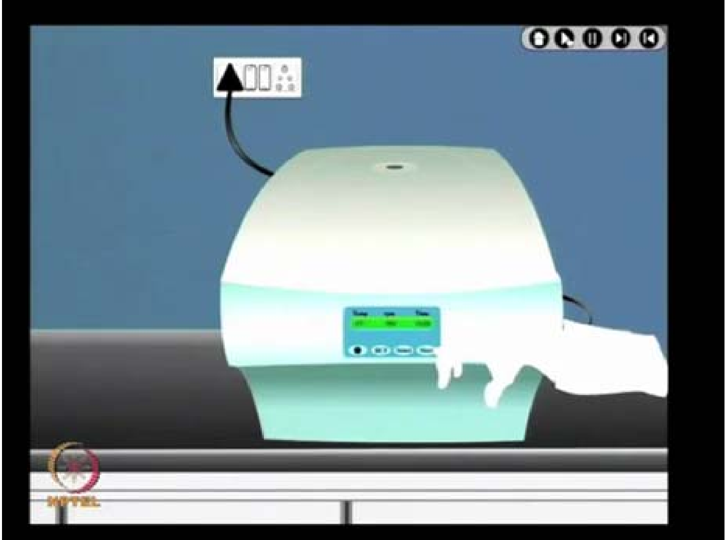

Continue growing the culture until it reaches the mid-exponential phase. The bacterial cells can be harvested by centrifuging the cells at 12000 rpm for 10 minutes at 4 degrees.

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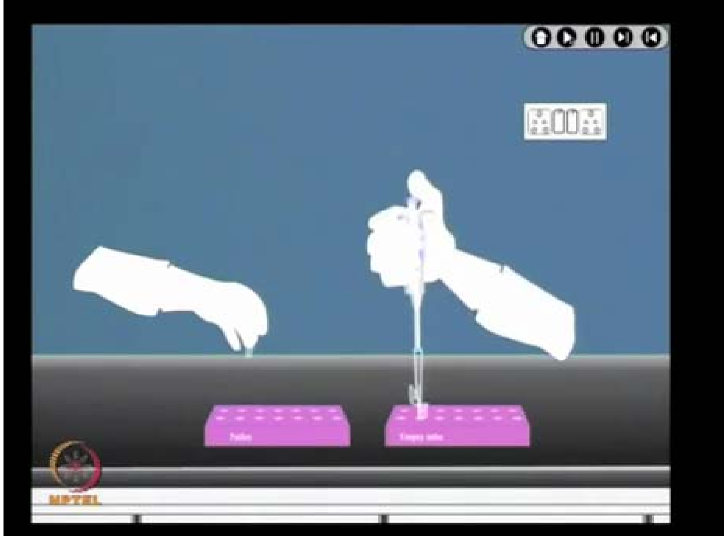

Wash the bacterial pellet with phosphate buffer of pH 7.4 for 4 times to remove the media

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Re-suspend the pellet with protease inhibitors and lysozymes and cells can be further ruptured by sonication in ice to prevent foaming and heat.

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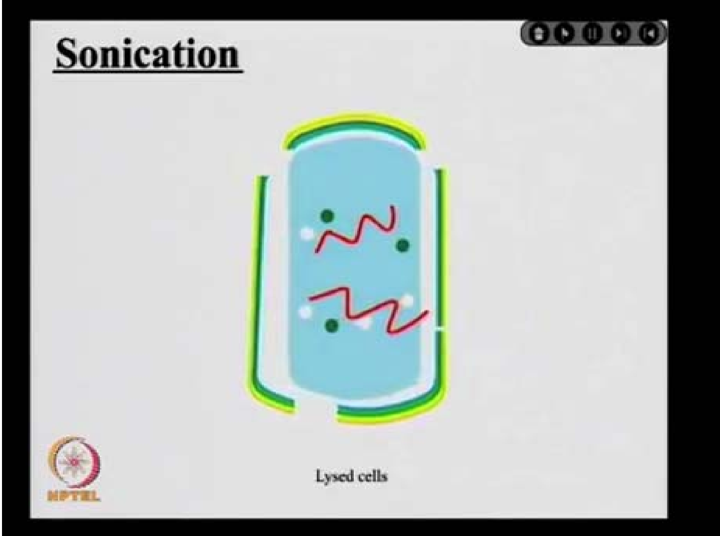

The sonication step helps to release the content of bacterial cells. Sonication can be performed by using a sonicator for 6 cycles of 5 second pulse with 30 seconds gap in between at 20% amplitude.

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Sonication involves the use of high energy sound waves that are capable of breaking open the outer membrane of the cells.

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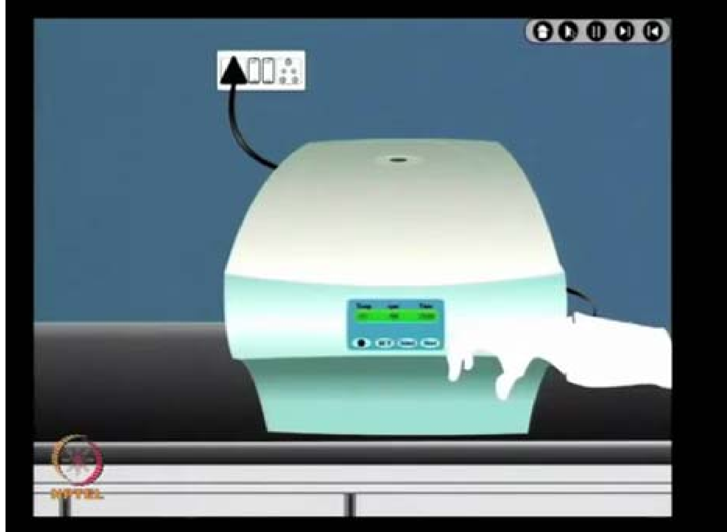

Cell debris and unbroken cells can be separated by the centrifugation step….

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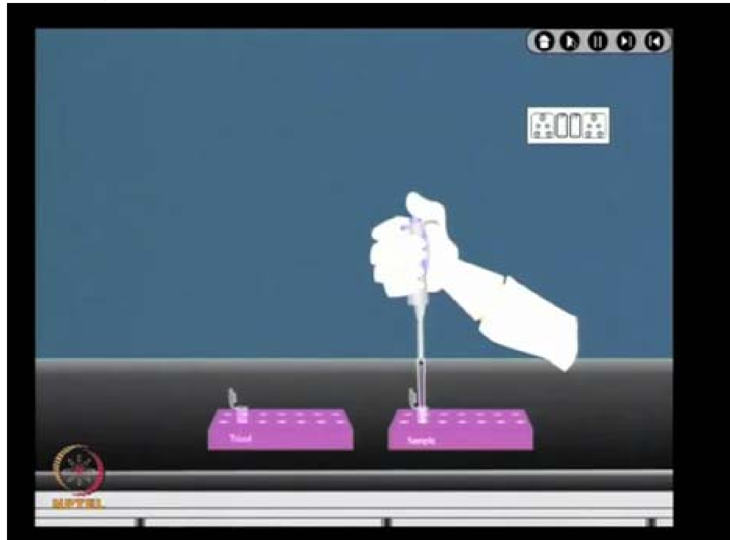

…to the supernatant add 1 ml of Trizol and 200 micro liter chloroform…

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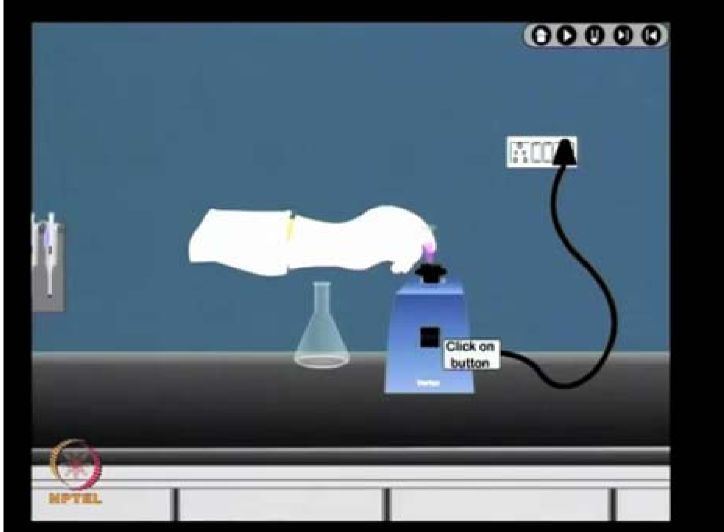

… and mix vigorously for 15 seconds so click on the Vortex here Mix it vigorously at room temperature…

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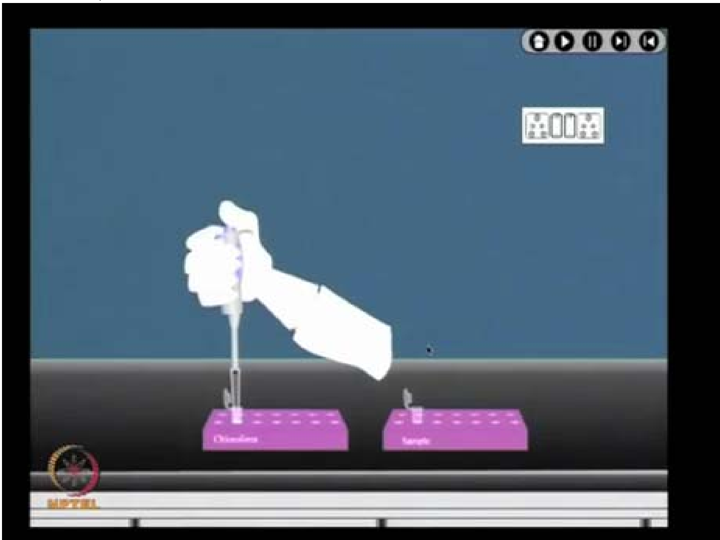

… to allow the phase separation

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After phase separation, centrifuge the sample at 12000 rpm…

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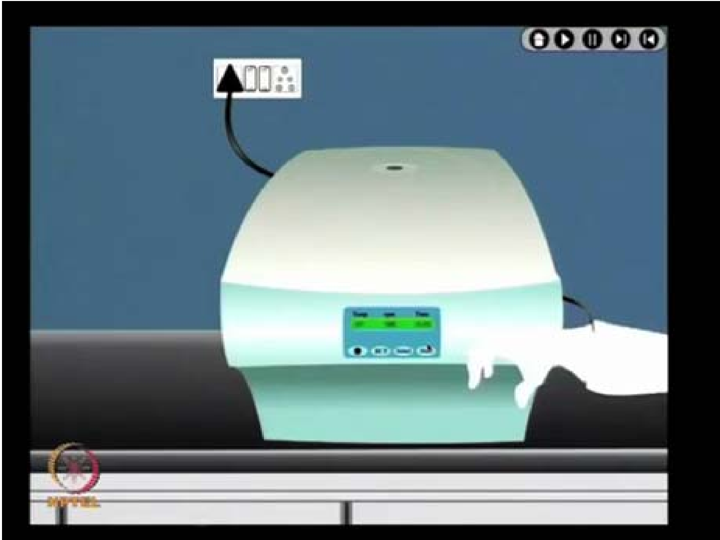

…for 15 minutes at 4 degrees.

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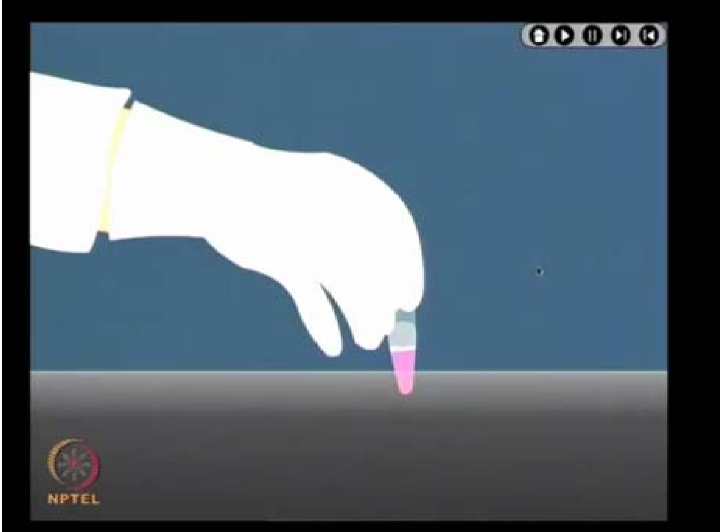

As I mentioned earlier, that different protocols one can use for bacterial proteome analysis. Here we are demonstrating the trizol method in which the top pale yellow layer contains RNA, the middle white precipitate having protein and lower phenol layer contains both protein and DNA.

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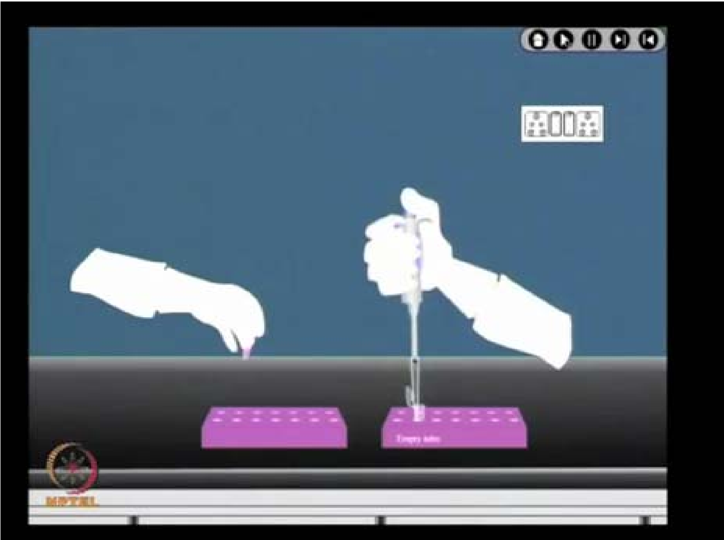

Remove the upper layer and use it for RNA isolation using isopropanol if you want to use RNA for some other applications.

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To the bottom layer add 300 micro liters of absolute alcohol per 1 ml of Trizol…

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… and mix gently to suspend the white precipitate and keep at room temperature for 3 minutes

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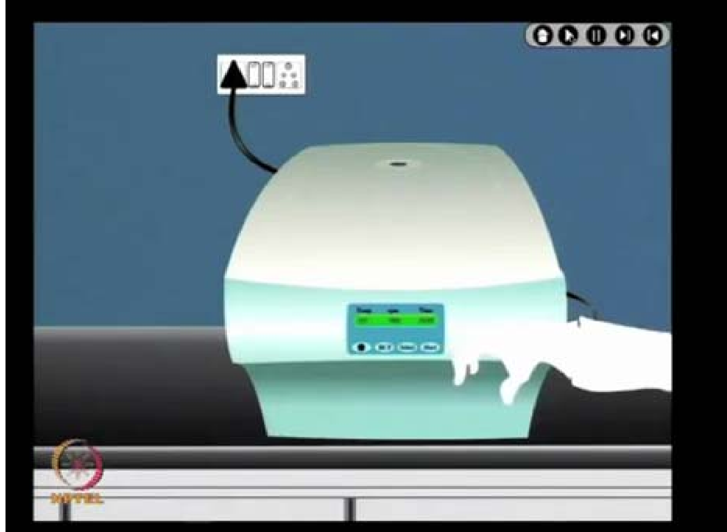

Centrifuge the mixture at 12000 rpm for 15 minutes at 4 degrees to precipitate the DNA.

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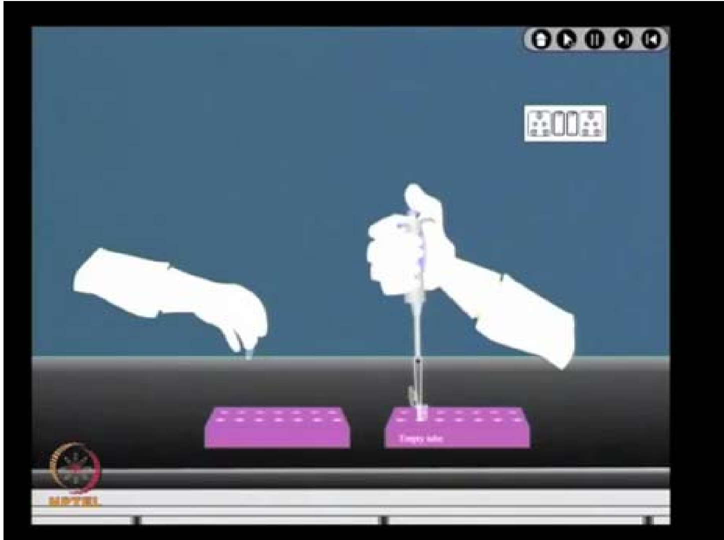

To the clear pink layer, add 4 volumes of chilled acetone and keep it at -20 degrees for 20 minutes.

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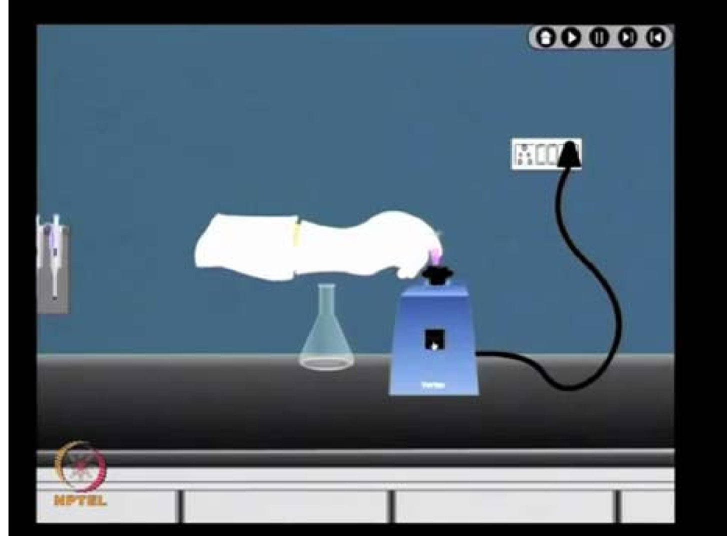

Protein pellet can be washed with 95% ethanol for 3 to 4 times. During the washing steps, you have to also Vortex so that the pellet is properly washed.

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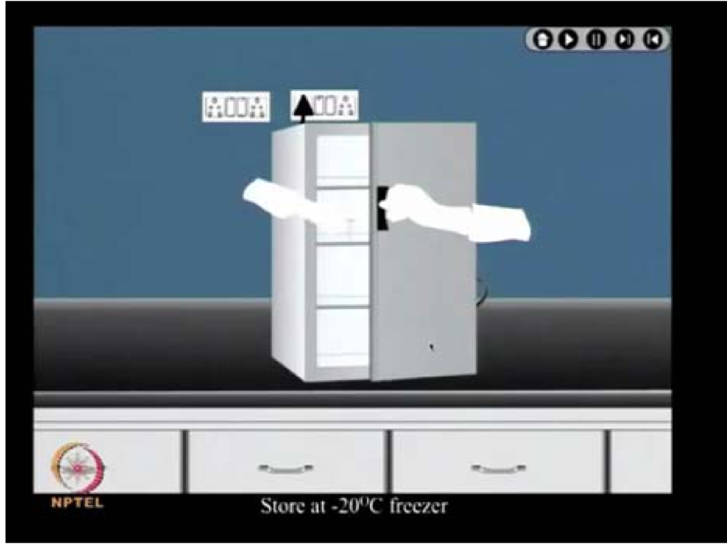

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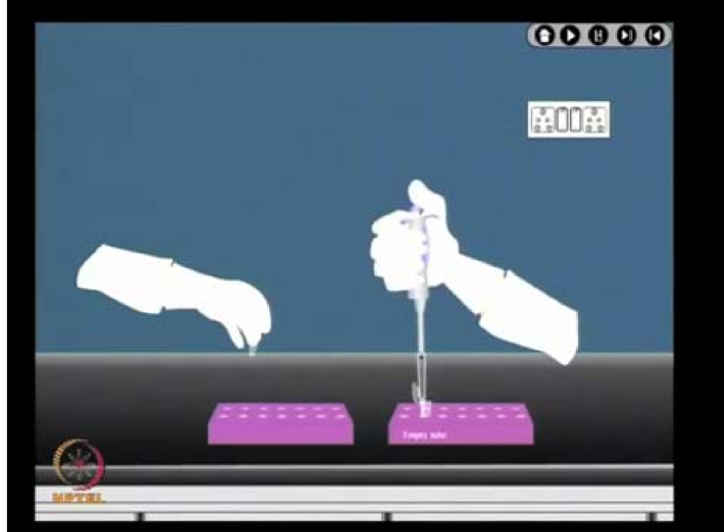

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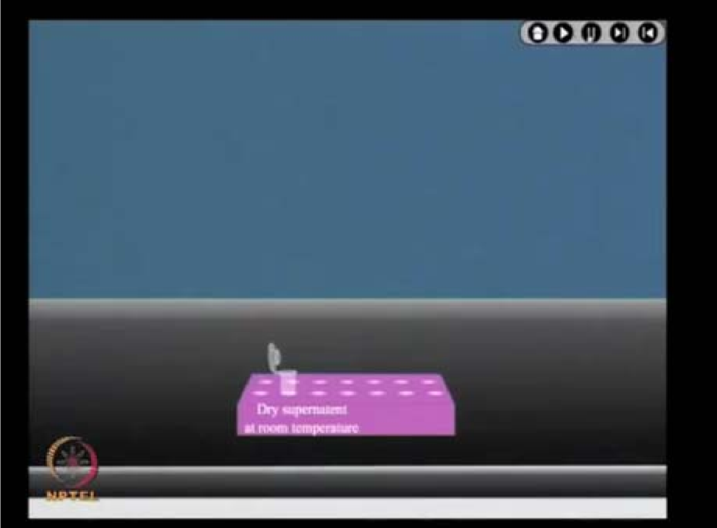

You need to allow the pellet to dry at the room temperature…

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…and then add the lysis buffer containing 7 molar of urea, 2 molar of thiourea….

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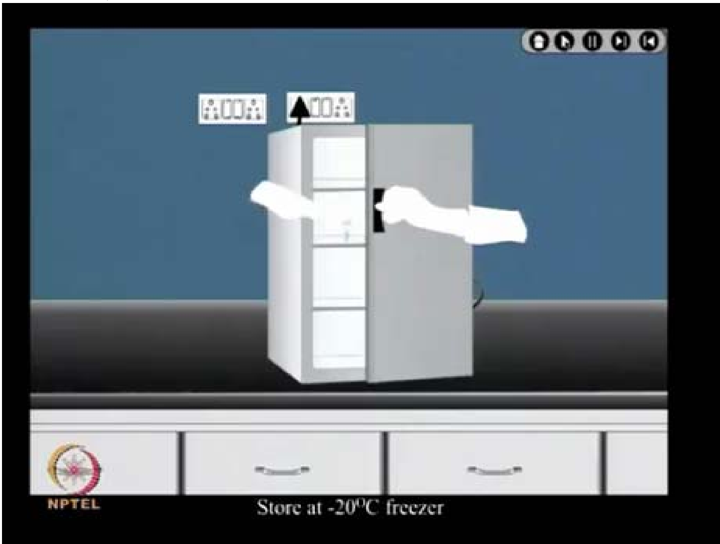

CHAPS, IPG buffer, DTT and No phenol blue. Ok, so I hope this animation was informative.

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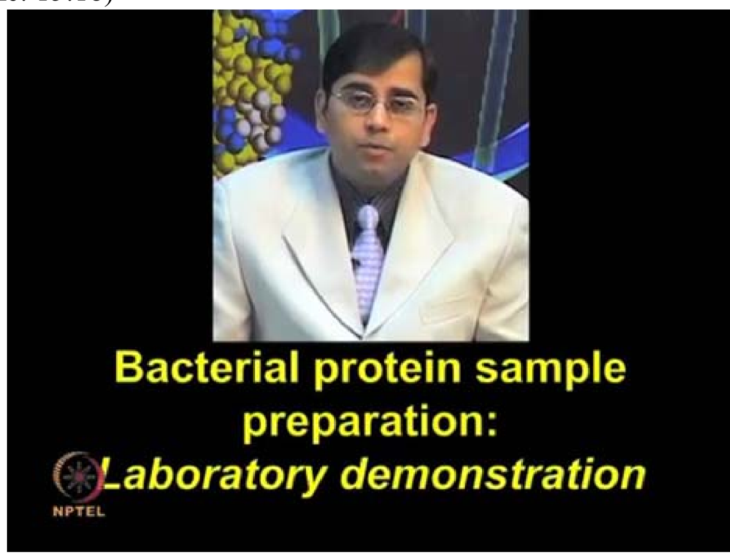

Bacterial protein extraction by using Trizol method and you must appreciate that in this method, you can obtain RNA, DNA and protein all the bio-molecules together.

So, to give you further information about the detailed protocol as well as how to perform these experiments in the lab, I will show you a video for the laboratory demonstration of bacterial protein sample preparation for proteomics applications. So let's watch this video.

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Bacterial proteins extraction

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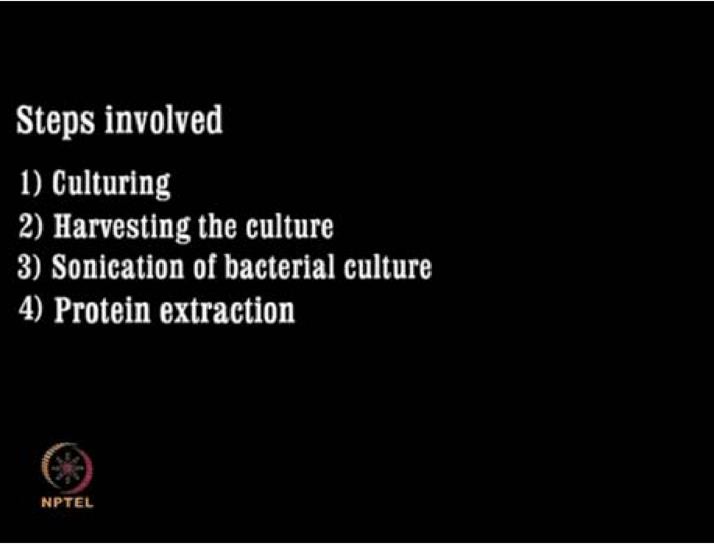

This process involves culturing of bacteria, harvesting and sonification of the obtained culture followed by protein extraction

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Bacterial culturing…

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Clean the laminar workspace thoroughly with ethanol and keep the master plate having bacterial culture ready

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Light the spirit lamp or Bunsen Burner to maintain sterile and aseptic conditions throughout the process.

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Carefully remove a bacterial colony from the master plate….

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…and inoculate…

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… the autoclaved growth media

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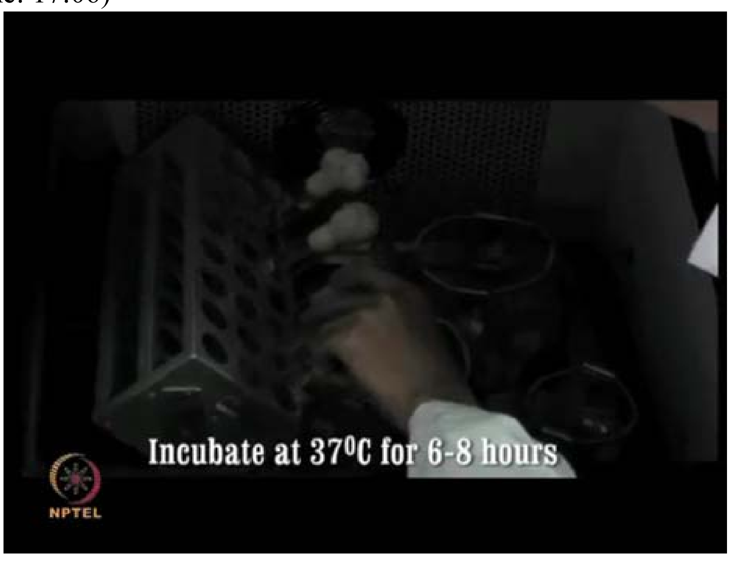

Incubate this inoculated sample at 37 degrees Celsius for 6 to 8 hours…

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…with constant shaking to allow the bacteria to grow; the turbidity of the culture which gradually increases indicates bacterial growth

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Culture harvesting

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Transfer the known bacterial culture to a fresh tube under sterile conditions.

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Centrifuge this tube…

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…at 12000 rpm for 10 minutes maintaining a temperature of 4 degrees Celsius

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Transfer the pellet obtained containing intact bacterial cells…

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…to a fresh, micro-centrifuged tube

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Wash this pellet thoroughly with phosphate buffer

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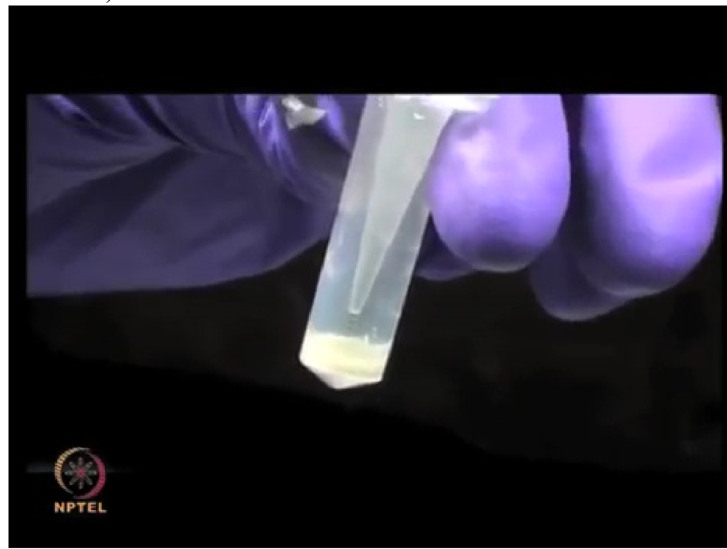

to remove any unwanted debris.

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Sonication

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The re-suspended pellet is sonicated on ice to enable the bacterial cells to break open ….

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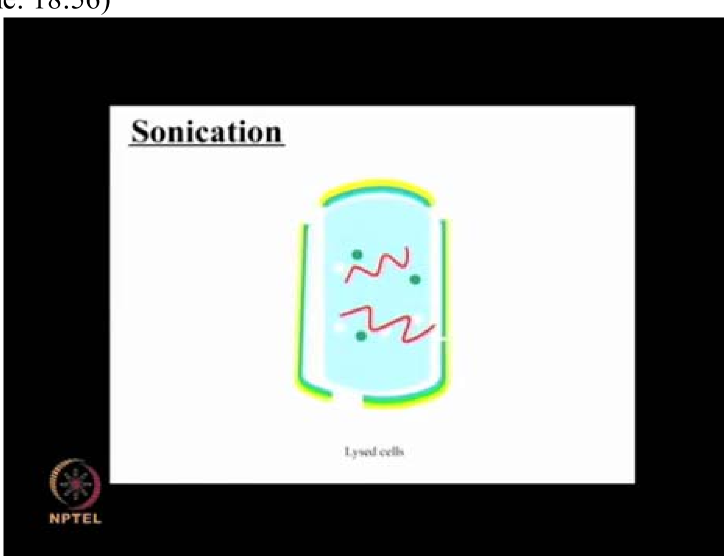

…so that its contents are released Sonication involves the use of high energy sound waves that are capable of breaking open the outer membranes of cells. All cellular contents including proteins of interest leak out of this disrupted membrane.

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Carry out the sonication procedure for 30 seconds…

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…with a pulse of 1 second at 40% amplitude

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Once it is complete, centrifuge the contents…

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And collect the supernatant that is obtained.

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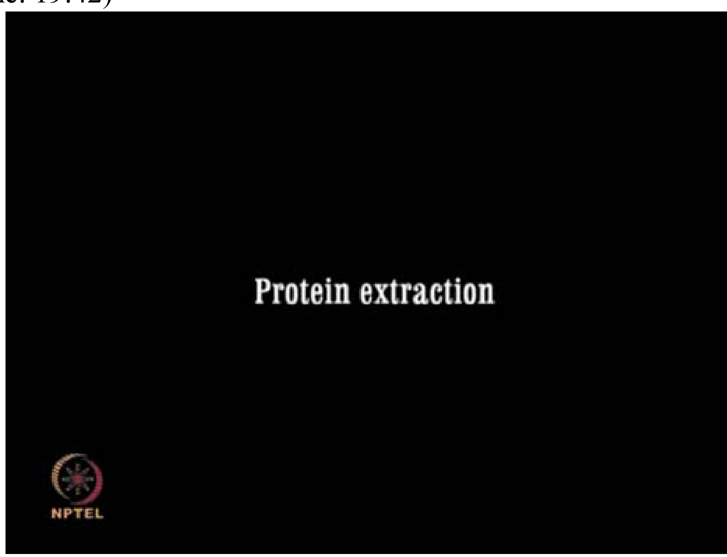

Protein Extraction

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The Trizol extraction protocol allows …

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…efficient separation of not just the bacterial proteins but also their DNA and RNA

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. Add the Trizol reagent consisting of guanidinium thiocyanate, phenol and chloroform to the supernatant obtained after sonication.

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Mix the contents thoroughly by Vortexing.

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Next, add chloroform to this solution, mix the contents…

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… And place the tube on ice for few minutes.

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Centrifuge the tube at 2000 rpm for 5 minutes.

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Three distinct layers will be obtained. The topmost is the aqueous layer containing RNA, the center is the interface containing proteins and the bottom layer is organic and consists of DNA

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Discard the transparent top layer having the RNA.

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Then add…

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Absolute alcohol to the remaining layers…

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… And mix the solutions well.

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Centrifuge the contents at 2000 rpm for 5 minutes.

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The DNA forms the white precipitate at the bottom of the tube while the proteins remain in the clear supernatant.

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Collect the supernatant in the fresh tube.

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Then add chilled acetone to this tube…

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… And mix well by Vortexing.

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Store the solution at -20 degrees Celsius for at least an hour…

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Before centrifuging it, to obtain the protein pellet…

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Discard the supernatant and….

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…dry the pellet at room temperature

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Reconstitute the dried pellet with rehydrated buffer and store overnight

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… at -20 degrees Celsius before carrying out protein quantification

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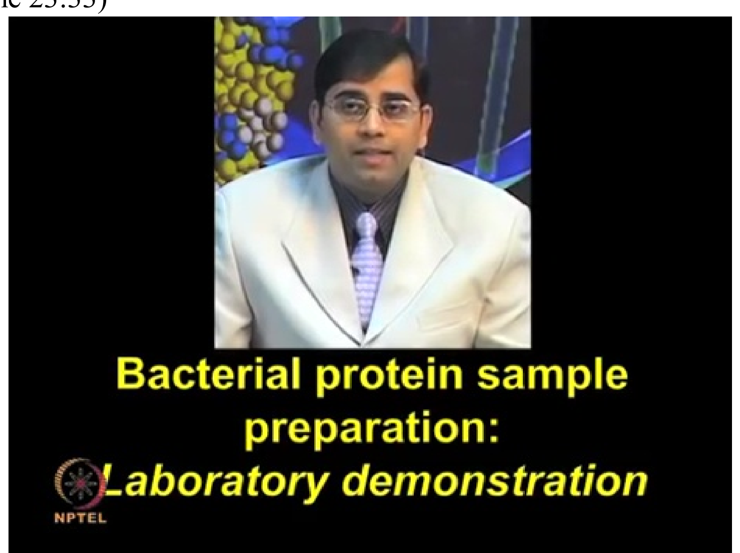

Ok, so I hope the video was informative and now you are able to appreciate the complexity involved in this procedure as well as how useful this method can be for different types of biomolecule extraction including DNA and RNA and how protein can be get rid of various types of contaminants by using Trizol method.

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Section V
Protein Quantification

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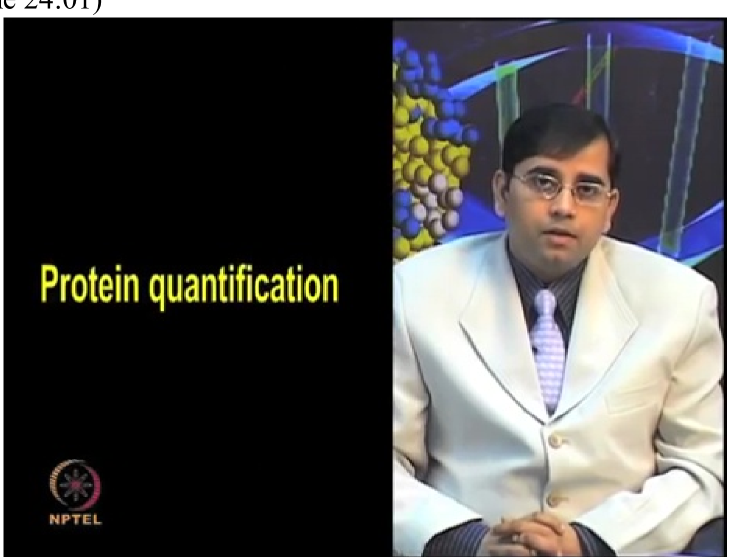

So now let's move on to the next topic which is protein quantification So by now you have prepared your protein sample by using different types of methods by keeping a very careful eye on different types of interfering substances and contaminants and you tried to eliminate all of those components

Now you want to know how much protein quantity you have in your sample whether you can perform your experiment with that protein or not. So, one need to know the protein quantity present in the protein sample. So there are different types of protein quantification methods are available. We will talk about protein quantification in detail now.

So there is another popular method which is Bradford Assay.

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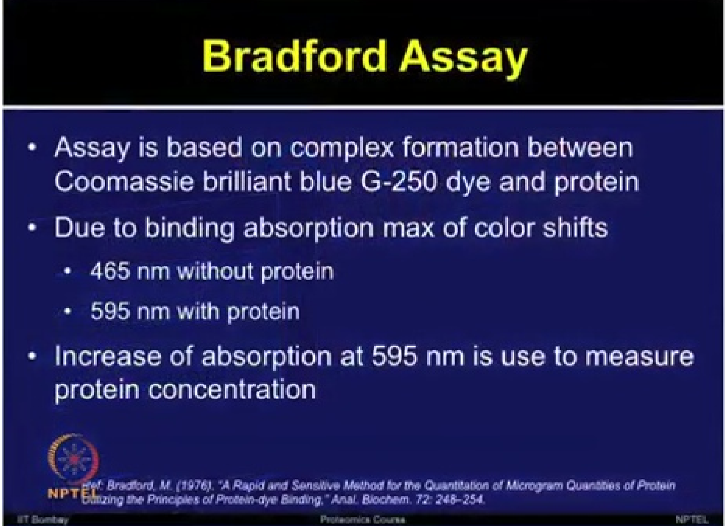

This Assay is based on the complex formation Coomassie dye G-250 and protein. Due to the binding absorption max, the color shifts from 465 nanometers to 595 nanometers. This increase of absorption at 550 595 nanometer is used to measure the protein concentration. The Bradford Assay has various advantages as compared to the Lowry or the BCA method….

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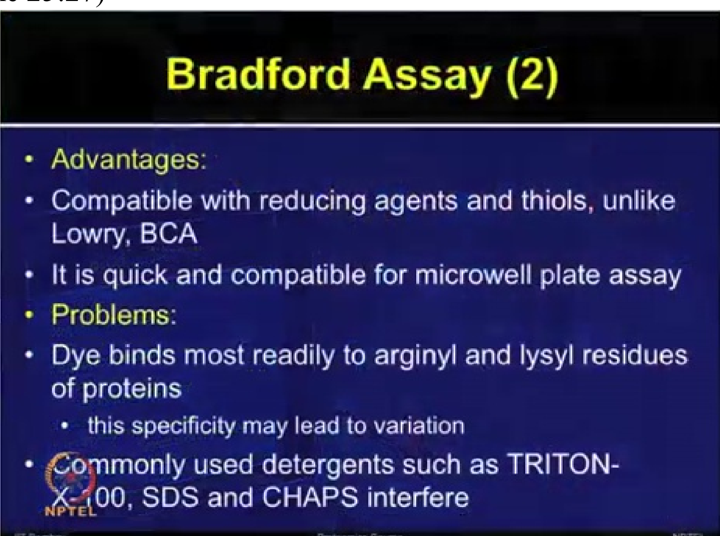

…because it is compatible with reducing agents and thiols This method is also very quick and it is compatible for the Micro well plate assays which can be performed in the 96 Well format

But there are different problems with the Bradford assay as well such as the dye binds are most readily to the ariginyl and lysyl residues of the protein and this specificity may lead to the variations. Now there are various detergents such as Triton X-100, SDS and CHAPS. They also interfere with the Bradford Assay.

So researchers have come up with various types of modified Bradford Assays for specific applications. Again you have to keep an eye what is the component of your buffer and you need to ensure that it is compatible with the Assay conditions or not.

So let's talk about the Bradford Assay in little bit more detail so that one can use that to determine protein concentration. If you want to perform this assay, what are the requirements? So first of all, you need one standard

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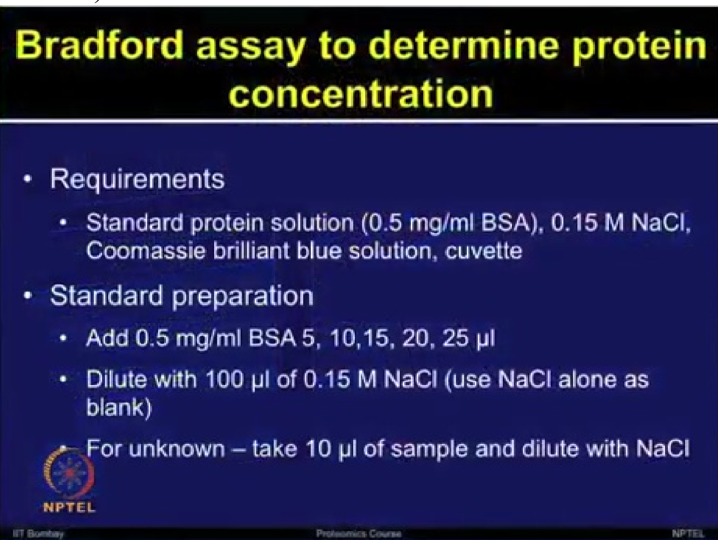

Let's say you can take BSA. You need some salt solution, Coomassie Brilliant blue solution and cuvette. So for standard preparation, you can take BSA and add different concentrations of this; 5, 10, 15, 20, 25 micro liters or you can go even more standard preparation series. Then dilute the sample with 0.15 molar of sodium chloride and total volume make it to 100 micro liters.

One sample where there is no protein there that can be used as the blank, which can be used to auto-zero the value in spectrophotometer as a blank and then further you can measure your samples.

For the unknown samples for which you want the protein concentration, you can take 10 or 15 micro liter of the sample and dilute with the sodium chloride. Then same treatment can be performed for this whole experiment.

Now if your unknown is giving you absorbance higher than your standard preparation, whatever series you have made, so it is better idea that you can try different dilutions of your unknown sample because it has to fall in the standard curve of the standard dilutions what you have taken.

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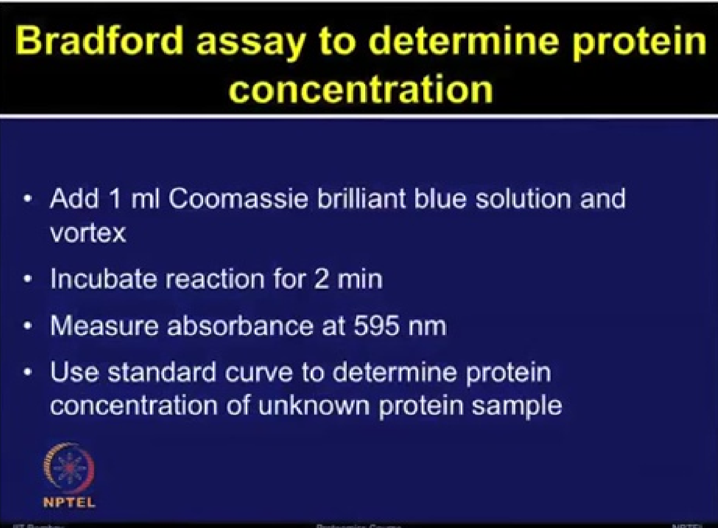

Now you can add 1 ml of Coomassie Brilliant blue solution and vortex it. After that one can incubate it for 2 minutes so that color can be developed and then it can be measured for absorbance at 595 nanometers.

Then you can use the standard curve to determine protein concentration of unknown protein sample. But often, once you plot your unknown protein sample, you may realize your values for the protein samples are either too low or too high to be plotted in the standard curve. So then you may need to adjust your unknown sample.

You may have to make dilution of it and use less volume or you may need to increase the volume of your sample so that it can fall in the range of the standard curve.

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Summary

Sample preparation workflow
Bacterial protein extraction
TRIzol protein extraction
Animation and lab demonstration
Protein Quantification

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